IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 3958
PASTAN et al.	Examiner: Zeman, Robert A.
Application No.: 09/673,707	Technology Center/Art Unit: 1645
Filed: January 11, 2001	DECLARATION OF DR. DAVID J. FITZGERALD
For: RECOMBINANT IMMUNOTOXIN DIRECTED AGAINST THE HIV-1 GP120 ENVELOPE GLYCOPROTEIN	
Customer No.: 20350	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, DR. DAVID J. FITZGERALD, hereby declare and state:

1. I received a B.A. in Microbiology from Trinity College, Dublin, Ireland, in 1977, and a Ph.D. in Microbiology from the University of Cincinnati, College of Medicine, Cincinnati, Ohio, in 1982.

2. Since receiving my doctorate in 1982, I have been a researcher in the Laboratory of Molecular Biology ("LMB") of the National Cancer Institute ("NCI"), of the U.S. National Institutes of Health ("NIH"). Since 1994, I have been Chief of the LMB's Biotherapy Section.

3. I am not an inventor on the captioned patent application, have no financial interest in it, and do not expect that any aspect of my employment at NIH will be affected by my submission of this Declaration.

4. I am an author or co-author of over 180 publications in the scientific literature. A copy of my c.v. is attached as Attachment 1.

5. I have been working in the field of targeting toxins to target cells since 1982. As reflected by the publications listed on my c.v., I have extensive experience in targeting toxins to target cells by attaching the toxins to targeting agents including (i) antibodies or fragments thereof (these hybrid molecule are known as "immunotoxins"), (ii) cytokines, and (iii) other molecules, including CD4, that are capable of targeting toxins to desired cell types. I have performed studies on aspects of each of these types of targeted toxins, and followed closely the results of using targeted toxins in clinical trials.

6. I am specifically knowledgeable about the attempts to develop targeted toxins of CD4-*Pseudomonas* exotoxin A ("PE") for use as therapeutic agents for HIV-1 infection, as reflected by the fact that I was a co-author on the first study on the use of a CD4-PE chimeric toxin to kill HIV-1 infected cells:

Chaudhary, V.K., Mizukami, T., Fuerst, T.R., **FitzGerald, D.J.**, Moss, B., Pastan, I., and Berger, E.A., "Selective killing of HIV-infected cells by recombinant human CD4-*Pseudomonas* exotoxin hybrid protein." Nature 335:369-372 (1988). A copy of the abstract of this publication is attached as Attachment 2 hereto.

7. My work in the pre-clinical development of CD4-PE toxin conjugates as a therapeutic to treat HIV-1 disease is further reflected by my co-authorship on the following publications:

i.) Berger, E.A., Clouse, K.A., Chaudhary, V.K., Chakrabarti, S., **FitzGerald, D.J.**, Pastan, I., and Moss, B., "CD4-*Pseudomonas* exotoxin hybrid protein blocks the spread of human immunodeficiency virus infection *in vitro* and is active against cells expressing the envelope glycoproteins from diverse primate immunodeficiency retroviruses. Proc. Natl. Acad. Sci. USA 86:9539-9543 (1989) A copy of the abstract of this publication is attached as Attachment 3 hereto.

ii.) Moss, B., Mizukami, T., Fuerst, T., Berger, E., Chaudhary, V., **FitzGerald, D.**, and Pastan, I., Localization of the HIV-binding region of CD4 and selective killing of HIV-infected cells with a hybrid CD4-*Pseudomonas* exotoxin. In: Girard, M. and

Valette, L. (Eds.). Colloque des Cent Gardes: Retroviruses of Human AIDS and Related Animal Diseases. France, Pasteur Vaccins, Marnes-La-Coquette, 1989, pp. 60-65.

iii.) Berger, E.A., Chaudhary, V.K., Clouse, K.A., **FitzGerald, D.J.**, Pastan, I., and Moss, B.:"Recombinant CD4-*Pseudomonas* exotoxin hybrid protein: Specific cytotoxic activity against T-cell lines infected with human immunodeficiency virus. In Groopman, J.E., Chen, I., Essex, M., and Weiss, R. (Eds.): Human Retroviruses, UCLA Symposia on Molecular and Cellular Biology, New Series, Vol 119. New York, Alan R. Liss, Inc., 1989, pp. 261-270.

iv.) Ashorn, P., Moss, B., Weinstein, J.N., Chaudhary, V.K., **FitzGerald, D.J.**, Pastan, I., and Berger, E.A, "Elimination of infectious HIV from human T-cell cultures by synergistic action of CD4-*pseudomonas* exotoxin and reverse transcriptase inhibitors." Proc. Natl. Acad. Sci. USA 87: 8889-8893 (1990) A copy of the abstract of this publication is attached as Attachment 4 hereto.

v.) Berger, E.A., Chaudhary, V.K., Clouse, K.A., Taraquemada, D., Nicholas, J.A., Rubino, K.L., **FitzGerald, D.J.**, Pastan, I., and Moss, B.: Recombinant CD4-*Pseudomonas* exotoxin hybrid protein displays HIV-specific cytotoxicity without affecting MHC class II-dependent functions. AIDS Res. Hum. Retroviruses 6: 795-804, 1990. A copy of the abstract of this publication is attached as Attachment 5 hereto.

vi.) Chaudhary, V.K., Moss, B., Berger, E.A., **FitzGerald, D.J.**, and Pastan, I.: CD4-PE40: A chimeric toxin active against HIV-infected cells. In Gallo, R.C. and Jay, G. (Eds.): The Human Retroviruses. Orlando, FL, Academic Press, 1991, pp. 379-387.

8. I followed closely the clinical trials of CD4-PE chimeric toxins. I am familiar with the results of those trials and with what persons of skill in the art believed following the failure of these chimeric toxins in those trials.

9. I am also familiar with the anti-gp120 antibody known as 3B3, as described in the specification of the captioned application. I am aware of the binding specificity and affinity of the Fv portion of this antibody and of the results of using immunotoxins composed of fusing the Fv portion of 3B3 (hereafter referred to as "3B3 Fv") to PE to kill cells infected with HIV-1.

10. I understand that the claims currently under examination in the captioned application are directed to immunotoxins having the binding specificity of 3B3 Fv and the binding affinity of 3B3 Fv, kits containing such immunotoxins, and compositions of these antibodies and a pharmaceutically acceptable carrier.

11. I understand that the Office Action dated April 13, 2006 (hereafter, "the Action"), regarding this application rejects the claims under examination as obvious over Matsushita et al., Aids Research Human Retroviruses 6(2):193-203 (1990) (hereafter, "Matsushita"), in view of Barbas, PNAS 91:3809-3813 (1994) and Pastan, U.S. Patent No. 5,458,878. I understand that the Matsushita reference relates to an anti-gp120 antibody known as 0.5β.

12. I understand that the counsel for the applicants has argued that the high hopes that might have been existed for the use of anti-gp120 antibodies at that time Matsushita was published in 1990 were dashed by the results of clinical trials of CD4-PE40 reported by Ramchandran et al., J. Infect Dis 170:1009-13 (1994) ("Ramachandran") and by Davey et al., J. Infect Dis 170:1180-8 (1994) ("Davey").

13. I understand that, on page 6, the Action states that the immunotoxin used in Ramanchandran and Davey are not analogous to the immunotoxins claimed in the claims under examination. I understand that the Action explains this conclusion as follows:

> " The immunotoxins of the instant invention . . . target cells expressing gp120 on their surface (i.e., infected cells) whereas the CD4-PE40 immunotoxin of Ramachandran et al. target any cell expressing CD4. Hence any 'results' based on the application of CD4-PE40 immunotoxin would not have any bearing on the perceived efficacy of immunotoxin based on the combination of the cited references. The same is true for the sCD[4]-PE immunotoxin disclosed by Davey et al."

(Technically, toxins targeted by non-antibody targeting molecules such as CD4 are not referred to in the art as "immunotoxins." Since the Action refers to the CD4-targeted toxins of Ramachandran and Davey as "immunotoxins," I will refer to them as such in this Declaration.)

14. The Action's position is factually untrue and would have been known to be false by a person of skill in the art as of the June 1998 filing date of the priority application. CD4 is a cell surface marker on the surface of certain cell types, B cells and macrophages that is bound by the gp120 protein of HIV-1. CD4 does not bind to itself. Neither the CD4-PE40 immunotoxin of Ramachandran nor the sCD4-PE immunotoxin of Davey would bind cells expressing CD4, as stated by the Action.

15. What the CD4-PE40 immunotoxin of Ramachandran and the sCD4-PE immunotoxin of Davey were intended to bind were cells infected by HIV-1, which express gp120 on their surface. The immunotoxins recited in the claims under examination have the binding affinity of the 3B3 Fv, which binds to the gp120 protein. Thus, both (i) the CD4-PE40 immunotoxin of Ramachandran and the sCD4-PE immunotoxin of Davey, and (ii) the immunotoxins of the present invention, bind to cells expressing gp120, not to cells expressing CD4. I and others in the art would therefore consider them to be analogous in terms of the cells they were intended to bind.

16. CD4 has interactions with major histocompatibility ("MHC") class 2 molecules. There was some intellectual concern at the time that CD4-PE toxins would bind to macrophages and other cells that express MHC class 2 molecules. This concern was tested pre-clinically, and found not be a concern well before the studies reported by Ramachandran and Davey. See, e.g., the Berger et al. (1990) publication listed above at ¶ 7, item (v).

17. I understand that, on page 6, the Action states:

"With regard to Point 4, contrary to Applicants assertion, CD4-P[E]40 immunotoxins would bind not only to cells expressing gp120, but also to any cell expressing CD4 on its surface."

This statement is factually untrue and would have been known to be false by a person of skill in the art as of the June 1998 filing date of the priority application. As already noted above, CD4 does not bind to itself. CD4-PE toxins do not bind "to any cell expressing CD4 on its surface." They do bind (and kill) cells expressing gp120 on their surface, as reported in my publications listed in paragraphs 6 and 7, above. And the only cells in the body that express gp120 are those infected with HIV-1.

17. I understand that, on page 6, the Action further states:

> "With regard to Point 5, since the CD4-PE40 immunotoxin would bind to any cell expressing CD4 on its surface, the hepatoxicity would logically be the result of said immunotoxin binding to healthy cells thereby disrupting some cellular or endocrine cascade present in man but not in the mouse."

This statement is factually untrue and would have been known to be false by a person of skill in the art as of the June 1998 filing date of the priority application. First, as already noted above, CD4 does not bind to itself. CD4-PE toxins do not bind "to any cell expressing CD4 on its surface." Second, cells in the liver (hepatocytes) do not express CD4. Thus, even if the Action was not incorrect about CD4-PE binding to CD4, the Action's argument would fail to explain the hepatoxicity observed in the human trials of CD4-PE toxins.

18. The above statements set forth my correction of the serious factual errors set forth in the Action. Not surprisingly, since the facts on which the Action are based are wrong, it also incorrectly presents what I and others of skill in the field understood following the results of the clinical trials of CD4-PE immunotoxins and before the filing of the captioned application.

19. At page 7, the Action states that the:

> "failure of a non-analogous immunotoxin [the CD4-PE immunotoxins] while it may have been discouraging would not necessarily remove the motivation provided by Matsushita, especially when his immunotoxin (which is analogous to the instant invention) was disclosed to have efficacy."

I consider this analysis to be incorrect, for several reasons.

(i) First, the CD4-targeted toxins, the 0.5β antibody of Matsushita, and the immunotoxins of the claims under examination all target cells expressing gp120, that is, HIV-1 infected cells. Matsushita's antibody is type-specific (see point (iv) below) and therefore binds only to cells infected by HIV-1 of the correct type, while both CD4 and the immunotoxins of the present invention would both bind to cells with less regard to the particular type of HIV-1 infecting the cells. But, to the extent that they are considered as binding to HIV-1 infected cells in preference to cells that are not infected by HIV-1, they would be considered analogous by persons of skill in the art.

(ii) Second, as noted above, liver cells do not express CD4. Thus, as noted above, even assuming that, contrary to fact, the CD4-PE immunotoxins would bind to cells expressing CD4, there would be no reason to think that the hepatoxicity observed in trials of CD4-PE immunotoxins would not also be found with respect to toxins targeted by the antibody of Matsushita.

(iii) Third, the Action comments that the antibody of Matsushita "was disclosed to have efficacy." The efficacy Matsushita discloses is that "toxin-conjugated anti-gp120 monoclonal antibody selectively killed HIV-infected cells in vitro." Matsushita, at page 199, second paragraph. Thus, the efficacy disclosed in Matsushita is similar to that disclosed in my publication in Nature two years earlier regarding the in vitro efficacy of CD4-PE in killing HIV-1 infected cells. See, Chaudhary et al., Nature 355:369-72 (1988). This would not by itself give persons of skill any reason to expect a different result with the 0.5β antibody of Matsushita than that found in clinical trials of CD4-PE toxins.

(iv) Fourth, Matsushita states that the 0.5β antibody is "type-specific." Matushiata, page 194, first line under heading "Antibody and immunotoxins." Matsushita notes in its discussion section that, while the binding activity of the 0.5β antibody was type specific, toxins conjugated to CD4 "also killed HIV-infected cells in vitro and were shown to be effective against [a] variety of divergent strains of HIV." Id., at page 200, second paragraph. Thus, Matsushita itself indicated the superiority of CD4 as a targeting agent against HIV-1 infected cells to the antibody the Matsushita authors themselves had developed. Any motivation Matsushita provided to create anti-env immunotoxins was removed by the failure of the CD4 toxins that the Matsushita authors themselves indicated were more broadly applicable than those the immunotoxin they had developed.

(v) Finally, both the immunotoxin of Matsushita and CD4-PE are toxins targeted to the envelope glycoprotein ("Env") of HIV-1.

For all of these reasons, even assuming that Matsushita provided a motivation to make Env-targeted toxins prior to the of the CD4-PE trials, I disagree with the Action's conclusion that Matsushita continued to provide such a motivation following the failure of those trials.

20. I note that the Action also states:

> With regard to Point 7, the "long felt need" for AIDS treatments was met by the teachings of Matsushita and would provide additional motivation for the skilled artisan to further refine the teachings of Matsushita.

Action, at page 7. I believe this statement is without merit. With respect, Matsushita's teachings did not "meet" the "long felt need" for AIDS treatments. Matsushita's 0.5β antibody-based immunotoxin is not only type-specific, but is also targeted to an epitope that even Matsushita admits is "within a highly variable region of gp120." Matsushita, at page 194, first full paragraph. Indeed, the Matsushita antibody proved unsuitable for clinical development since the site it binds is one the HIV-1 virus readily mutates so that infected cells do not express the epitope bound by the antibody. I am not aware, some 16 years after the publication of Matsushita in 1990, that the Matsushita 0.5β antibody was ever brought into pre-clinical development. It clearly did not "meet the long-felt need" for an AIDS treatment, as asserted by the Action. In contrast, 3B3Fv-targeted immunotoxins of the claims are continuing to be successful in pre-clinical studies, including one designed to see if the immunotoxin would induce the same hepatoxicity as that seen in the CD4-PE trials referenced above. See, Kennedy et al., J Leukoc Biol (August 2006). A copy of the abstract of this publication is attached as Attachment 6 hereto.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these

statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Oct 13th, 2006
Date

David J. FitzGerald
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CURRICULUM VITAE

Name David J. FitzGerald

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Education

Trinity College, Dublin, Ireland BA Mod 1977 Microbiology
U of Cincinnati, Col of Med, OH PhD 1982 Microbiology

Employment

1982 - 1984	Staff Fellow, LMB, DCBDC, NCI, NIH
1985 - 1987	Senior Staff Fellow, LMB, DCBDC, NCI, NIH
1987 - 1994	Microbiologist, LMB, DCBDC, NCI, NIH
1994-present	Chief, Biotherapy Section, LMB, DBS, NCI, NIH

Honors

January 1980	Awarded the Albert J. Ryan Fellowship.
June 1991	NIH Director's Award
June 1992	Pierce Immunotoxin Award, at The Third International Immunotoxin Meeting, Orlando, FL.
July 1994	Chair, Gordon Conference, *Drug Carriers in Medicine & Biology.*
September 1995	NIH Award of Merit
February 1999	Awarded NCI Intramural Research Award (IRA)
September 2004	NIH Merit Award
July 2002	NIAID Biodefense Grant Award

Teaching Experience

Was invited to teach a two-week (September - October, 1988) course on immunotoxins at the Shanghai Institute of Biochemistry (joint U.S. National Academy of Science and Chinese Academy of Science program).

Editorial Boards

Infection and Immunity (1987-1989)
Journal of National Cancer Institute (1990-1994)
Journal of Pharmaceutical Sciences
Journal of Bioconjugate Chemistry (1990-1994)
Journal of Drug Targeting
Therapeutic Immunity
Journal of Biological Chemistry (1996-)

Peer Review Experience

Member of Study Section for Tropical Medicine and Parasitology, October 1986
Member of special study section to review toxin-based grant proposals, July 1988
American Cancer Society, Ad Hoc Reviewer for Immunotherapy Study Section, Spring 1991

Clinical Investigation

Co-investigator on FDA-approved protocol with PE-ANTI-TAC to treat patients with adult-T-cell leukemia, IND #BB IND 2174 (NSC 600665).

Co-investigator on FDA-approved protocol with OVB3-PE to treat patients with ovarian cancer, IND #IND2688 (NSC 615048).

Co-investigator on FDA-approved protocol with LMB-1 to treat patients with adenocarcinomas IND #5017 (NSC 651311).

Co-investigator on Phase I application for IND of immunotoxin directed to CD22+ leukemias and lymphomas (IND/NSC numbers not yet available).

Committee Experience

An original member and presently serving on NCI's "Technology Review Group". Responsible for reviewing all new invention reports and making strategic decisions about how to prosecute NCI's existing patent portfolio.

Societies

AAAS
American Society for Biochemistry and Molecular Biology

Patents

Pastan, I., Willingham, M.C., and FitzGerald, D.J.: *Pseudomonas* exotoxin conjugate immunotoxins. (Assignee: U.S.A., D.H.H.S.) (Filed January 26, 1984.) Granted U.S. Patent #4,545,985, October 8, 1985.

Pastan, I., FitzGerald, D.J.P., and Willingham, M.C.: Monoclonal antibody against ovarian cancer cells (OVB3). Patent #4,806,494, February 21, 1989.

Pastan, I., Adhya, S., and FitzGerald, D.J.P.: Recombinant *Pseudomonas* exotoxin: Construction of an active immunotoxin with low side effects. Patent #4,892,827, January 9, 1990.

Bjorn, M.J., FitzGerald,D.J., Frankel, A.E.,Laird, W.J., Pastan, I.H., Ring, D.B., Willingham, M. C., and Windelhake, J. L.: Anti-human ovarian cancer immunotoxins and methods of use thereof. (Assignee: Cetus Corporation) (Filed July 6, 1987.) Granted U.S. Patent #4,958,009, September 18, 1990.

Pastan, I., FitzGerald, D., and Ogata, M.: Selectively cytotoxic IL-4-PE40 fusion protein. (Assignee: U.S.A., D.H.H.S.) (Filed May 12, 1989.) Granted U.S. Patent #5,082,927, January 21, 1992.

Berger, E.A., Fuerst, T.R., Pastan, I., FitzGerald, D., Mizukami, T., and Chaudhary, V.K.: CD-4/cytotoxic gene fusions. Patent #5,206,353, (Assignee: U.S.A., D.H.H.S.) (Filed July 22, 1988.) Granted U.S. Patent #5,206,353, April 27, 1993.

Pastan, I.H., Trevor, P., FitzGerald, D.J., Debinski, W., and Siegall, C.: Recombinant chimeric proteins deliverable across ellular membranes into cytosol of target cells. (Assignee: U.S.A., D.H.H.S.) (Filed March 4, 1991.) Granted U.S. Patent #5,328,984, July 12, 1994.

Berger, E.A., Moss, B., Fuerst, T.R., Pastan, I., FitzGerald, D., Mizukami, T., and Chaudhary, V.K.: Cytotoxic agent against specific virus infection. (Assignee: U.S.A.) (Filed February 25, 1993.) Granted U.S. Patent #5,428,143, June 27, 1995.

Pastan, I., Chaudhary, V.K., and FitzGerald, D.: P. exotoxin fusion proteins have COOH-terminal alterations which increase cytotoxicity. (Assignee: U.S.A., D.H.H.S.) (Filed May 14, 1990.) Granted U.S. Patent #5,458,878, October 17, 1995.

Pastan, I., FitzGerald, D., and Chaudhary, V.K.: Pseudomonas exotoxins (PE) and conjugates thereof having lower animal toxicity with high cytocidal activity through substitution of positively charged amino acids. (Assignee: U.S.A., D.H.H.S.) (Filed October 1, 1993.) Granted U.S. Patent #5,512,658, April 30, 1996.

BIBLIOGRAPHY

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2. FitzGerald, D.J.P., Morris, R.E., and Saelinger, C.B.: The essential role of calcium in cellular internalization of *Pseudomonas* toxin. *Infect. Immun.* 35: 715-720, 1982.

3. FitzGerald, D.J.P., Padmanabhan, R., Pastan, I., and Willingham, M.C.: Adenovirus-induced release of epidermal growth factor and *Pseudomonas* toxin into the cytosol of KB cells during receptor-mediated endocytosis. *Cell* 32: 607-617, 1983.

4. FitzGerald, D.J.P., Trowbridge, I.S., Pastan, I., and Willingham, M.C.: Enhancement of toxicity of antitransferrin receptor antibody *Pseudomonas* exotoxin conjugates by adenovirus. *Proc. Natl. Acad. Sci. USA* 80: 4134-4138, 1983.

5. Willingham, M.C., Haigler, H.T., FitzGerald, D.J.P., Gallo, M.G., Rutherford, A.V., and Pastan, I.: The morphologic pathway of binding and internalization of epidermal growth factor in cultured cells. *Exp. Cell Res.* 146: 163-175, 1983.

6. Zoon, K.C., Arnheiter, H., Zur Nedden, D., FitzGerald, D.J.P., and Willingham, M.C.: Human interferon alpha enters cells by receptor-mediated endocytosis. *Virology* 130: 195-203, 1983.

7. FitzGerald, D.J.P., Morris, R.E., and Saelinger, C.B.: Inhibition of *Pseudomonas* toxin internalization by methylamine. *Rev. Infec. Dis.* 5: Suppl. S985-991, 1983.

8. FitzGerald, D.J.P., Waldmann, T.A., Pastan, I., and Willingham, M.C.: PE-anti-Tac: a cell-specific immunotoxin active against cells expressing the human T-cell growth factor receptor. *J. Clin. Invest.* 74: 966-971, 1984.

9. Seth, P., FitzGerald, D.J.P., Willingham, M.C., and Pastan, I.: Role of a low pH environment in adenovirus enhancement of the toxicity of a *Pseudomonas* exotoxin epidermal growth factor conjugate. *J. Virol.* 51: 650-655, 1984.

10. Akiyama, S., Gottesman, M.M., Hanover, J.A., FitzGerald, D.J.P., Willingham, M.C., and Pastan, I.: Verapamil enhances the toxicity of an epidermal growth factor *Pseudomonas* exotoxin conjugate. *J. Cell Physiol.* 120: 271-279, 1984.

11. Seth, P., FitzGerald, D., Ginsberg, H., Willingham, M., and Pastan, I.: Evidence that the penton base of adenovirus is involved in potentiation of *Pseudomonas* exotoxin conjugated to epidermal growth factor. *Mol. Cell. Biol.* 4: 1528-1533, 1984.

12. Akiyama, S., Seth, P., Pirker, R., FitzGerald, D., Gottesman, M.M., and Pastan, I.: Potentiation of cytotoxic activity of immunotoxins on cultured human cells. *Cancer Res.* 45: 1005-1007, 1985.

13. Pirker, R., FitzGerald, D.J., Hamilton, T.C., Ozols, R.F., Willingham, M.C., and Pastan, I.: Anti-transferrin receptor antibody linked to *Pseudomonas* exotoxin as a model immunotoxin in human ovarian carcinoma cell lines. *Cancer Res.* 45: 751-757, 1985.

14. FitzGerald, D.J.P.: Transport of adenovirus and toxin conjugates into cells via the common pathway of receptor-mediated endocytosis. *Microbiology* 85-90, 1985.

15. Pirker, R., FitzGerald, D.J., Hamilton, T.C., Ozols, R.F., Laird, W., Frankel, A.E., Willingham, M.C., and Pastan, I.: Characterization of immunotoxins active against ovarian cancer cell lines. *J. Clin. Invest.* 76: 1261-1267, 1985.

16. Zoon, K.C., Arnheiter, H., Zur Nedden, D., FitzGerald, D.J., and Willingham, M.C.: Procedures for measuring receptor-mediated binding and internalization of human interferon. In *Methods of Enzymology* 119: 332-339, 1986.

17. Pastan, I., Seth, P., FitzGerald, D., and Willingham, M.C.: Adenovirus entry into cells: Some new observations on an old problem. In Notkins, A.L. and Oldstone, M.B.A. (Eds.): *Concepts in Viral Pathogenesis*, Vol. II. New York, Springer Verlag, 1986, pp. 141-146.

18. FitzGerald, D.J., Willingham, M.C., and Pastan, I.: Anti-tumor effects of an immunotoxin made with *Pseudomonas* exotoxin in a nude mouse model of human ovarian cancer. *Proc. Natl. Acad. Sci. USA* 83: 6627-6630, 1986.

19. Pastan, I., Willingham, M.C., and FitzGerald, D.J.P.: Immunotoxins. *Cell* 47: 641-648, 1986.

20. Seth, P., FitzGerald, D., Willingham, M.C., and Pastan, I.: Pathway of adenovirus entry into cells. In Crowell, R. and Lonberg-Holm, K. (Eds.): *Virus Attachment and Entry Into Cells*. Washington, D.C., American Society for Microbiology, 1986, pp. 191-195.

21. Hwang, J., FitzGerald, D.J.P., Adhya, S., and Pastan, I.: Functional domains of *Pseudomonas* exotoxin identified by deletion analysis of the gene expressed in *E. coli*. *Cell* 48: 129-136, 1987.

22. FitzGerald, D.J.P., Bjorn, M.J., Ferris, R.J., Winkelhake, J.L., Frankel, A.E., Hamilton, T.C., Ozols, R.F., Willingham, M.C., and Pastan, I.: Antitumor activity of an immunotoxin in a nude mouse model of human ovarian cancer. *Cancer Res.* 47: 1407-1410, 1987.

23. Willingham, M.C., FitzGerald, D.J., and Pastan, I.: *Pseudomonas* exotoxin coupled to a monoclonal antibody against ovarian cancer inhibits the growth of human ovarian cancer cells in a mouse model. *Proc. Natl. Acad. Sci. USA* 84: 2474-2478, 1987.

24. FitzGerald, D.J., Willingham, M.C., Cardarelli, C.O., Hamada, H., Tsuruo, T., Gottesman, M.M., and Pastan, I.: A monoclonal antibody *Pseudomonas* toxin conjugate that specifically kills multidrug-resistant cells. *Proc. Natl. Acad. Sci USA* 84: 4288-4292, 1987.

25. Lyall, R.M., Hwang, J., Cardarelli, C., FitzGerald, D., Akiyama, S.-I., Gottesman, M.M., and Pastan, I.: Isolation of human KB cell lines resistant to epidermal growth factor *Pseudomonas* exotoxin conjugates. *Cancer Res.* 47: 2961-2966, 1987.

26. Chaudhary, V.K., FitzGerald, D.J., Adhya, S., and Pastan, I.: Activity of a recombinant fusion protein between transforming growth factor type α and *Pseudomonas* toxin. *Proc. Natl. Acad. Sci. USA* 84: 4538-4542, 1987.

27. Cryz, S.J., Jr., Furer, E., Sadoff, J.C., Germanier, R., Pastan, I., Willingham, M.C., and FitzGerald, D.J.P.: The use of *Pseudomonas aeruginosa* toxin A in the construction of conjugate vaccines and immunotoxins. *Rev. Infect. Dis.* 9: S644-749, 1987.

28. Pirker, R., FitzGerald, D., Willingham, M.C., and Pastan, I.: Immunotoxins and endocytosis. *Lymphokines* 14: 361-382, 1987.

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1: Nature. 1988 Sep 22;335(6188):369-72.

nature

Selective killing of HIV-infected cells by recombinant human CD4-Pseudomonas exotoxin hybrid protein.

Chaudhary VK, Mizukami T, Fuerst TR, FitzGerald DJ, Moss B, Pastan I, Berger EA.

Laboratory of Molecular Biology, National Cancer Institute, Bethesda, Maryland 20892.

It is projected that in the absence of effective therapy, most individuals infected with human immunodeficiency virus (HIV) will develop acquired immune deficiency syndrome (AIDS) and ultimately succumb to a combination of opportunistic microbial infections, malignancies and direct pathogenic effects of the virus. Anti-viral agents, immunomodulators, and inhibitors of specific HIV functions are being tested as potential treatments to alleviate the high morbidity and mortality. An alternative therapeutic concept involves the development of cytotoxic agents that are targeted to kill HIV-infected cells. Here we describe the purification and characterization of a recombinant protein produced in Escherichia coli that contains the HIV-binding portion of the human CD4 molecule linked to active regions of Pseudomonas exotoxin A. This hybrid protein displays selective toxicity toward cells expressing the HIV envelope glycoprotein and thus represents a promising novel therapeutic agent for the treatment of AIDS.

PMID: 2843774 [PubMed - indexed for MEDLINE]

Exhibit 2

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1: Proc Natl Acad Sci U S A. 1989 Dec;86(23):9539-43.

CD4-Pseudomonas exotoxin hybrid protein blocks the spread of human immunodeficiency virus infection in vitro and is active against cells expressing the envelope glycoproteins from diverse primate immunodeficiency retroviruses.

Berger EA, Clouse KA, Chaudhary VK, Chakrabarti S, FitzGerald DJ, Pastan I, Moss B.

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20852.

We previously described an unusual recombinant protein, designated CD4(178)-PE40, containing the gp120 binding region of human CD4 linked to active regions of Pseudomonas exotoxin A. The ability of this molecule to selectively inhibit protein synthesis in cells expressing the surface envelope glycoprotein of human immunodeficiency virus (HIV) suggested this molecule may be useful in treating infected individuals. To further evaluate its therapeutic potential, several in vitro properties of this hybrid toxin were examined. CD4(178)-PE40 was found to be an extremely potent cytotoxic agent, selectively killing HIV-infected cells with IC50 values around 100 pM. In a coculture system employing mixtures of HIV-infected and -uninfected cells, the hybrid toxin inhibited spread of the infection, as judged by a delay in HIV-induced cell killing and a dramatic suppression of free virus production. Experiments with control recombinant proteins indicated that this protective effect was primarily due to selective killing of the HIV-infected cells, rather than to a simple blocking effect of the CD4 moiety of the hybrid toxin. Using recombinant vaccinia viruses as expression vectors, we found the hybrid toxin to be active against cells expressing the envelope glycoproteins of divergent isolates of HIV-1, as well as HIV-2 and simian immunodeficiency virus. These results provide further support for the therapeutic potential of CD4(178)-PE40 in the treatment of HIV-infected individuals.

Related Links

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1: Proc Natl Acad Sci U S A. 1990 Nov;87(22):8889-93.

Elimination of infectious human immunodeficiency virus from human T-cell cultures by synergistic action of CD4-Pseudomonas exotoxin and reverse transcriptase inhibitors.

Ashorn P, Moss B, Weinstein JN, Chaudhary VK, FitzGerald DJ, Pastan I, Berger EA.

Laboratory of Viral Diseases, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

We have previously described a recombinant protein, designated CD4(178)-PE40, consisting of the human immunodeficiency virus (HIV) envelope glycoprotein-binding region of human CD4 linked to the translocation and ADP-ribosylation domains of Pseudomonas aeruginosa exotoxin A. By virtue of its affinity for gp120 (the external subunit of the HIV envelope glycoprotein), the hybrid toxin selectively binds to and kills HIV-1-infected human T cells expressing surface envelope glycoprotein and also inhibits HIV-1 spread in mixed cultures of infected and uninfected cells. We now report that CD4(178)-PE40 and reverse transcriptase inhibitors exert highly synergistic effects against HIV-1 spread in cultured human primary T cells. Furthermore, combination treatment can completely eliminate infectious HIV-1 from cultures of human T-cell lines. This conclusion is based on protection of a susceptible cell population from HIV-induced killing, complete inhibition of virus protein accumulation, and elimination of HIV DNA (as judged by quantitative polymerase chain reaction analysis). The results highlight the therapeutic potential of treatment regimens involving combination of a virostatic drug that inhibits virus replication plus an agent that selectively kills HIV-infected cells.

PMID: 1701055 [PubMed - indexed for MEDLINE]

Exhibit 4

1: AIDS Res Hum Retroviruses. 1990 Jun;6(6):795-804.

Recombinant CD4-Pseudomonas exotoxin hybrid protein displays HIV-specific cytotoxicity without affecting MHC class II-dependent functions.

Berger EA, Chaudhary VK, Clouse KA, Jaraquemada D, Nicholas JA, Rubino KL, Fitzgerald DJ, Pastan I, Moss B.

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892.

The present study describes several in vitro activities of CD4(178)-PE40, a recombinant protein containing a portion of human CD4 linked to active regions of Pseudomonas aeruginosa exotoxin A. Using assays for cell viability, we demonstrate that the hybrid toxin displays highly selective cytotoxicity for HIV-infected T lymphocytes. In a latently infected human T-cell line which is inducible for HIV expression, toxin sensitivity is observed only upon virus induction. At concentrations which readily kill HIV-infected T cells, CD4(178)-PE40 has no observable cytotoxic effects on uninfected human cell lines expressing surface major histocompatibility complex (MHC) Class II molecules, and does not interfere with cellular responses known to be dependent on functional association between CD4 and MHC Class II molecules.

PMID: 2114147 [PubMed - indexed for MEDLINE]

Exhibit 5

1: J Leukoc Biol. 2006 Aug 21; [Epub ahead of print]

Anti-HIV-1 immunotoxin 3B3(Fv)-PE38: enhanced potency against clinical isolates in human PMBCs and macrophages, and negligible hepatotoxicity in macaques.

Kennedy PE, Bera TK, Wang QC, Gallo M, Wagner W, Lewis MG, Berger EA, Pastan I.

*Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases and Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland; and Southern Research Institute, Frederick, Maryland.

Highly active antiretroviral therapy (HAART) against human immunodeficiency virus type 1 (HIV-1) infection dramatically suppresses viral load, leading to marked reductions in HIV-1 associated morbidity and mortality. However, infected cell reservoirs and low-level replication persist in the face of suppressive HAART, leading invariably to viral rebound upon cessation of treatment. Toxins engineered to target the Env glycoprotein on the surface of productively infected cells represent a complementary strategy to deplete these reservoirs. We described previously highly selective killing of Env-expressing cell lines by CD4(178)-PE40 and 3B3(Fv)-PE38, recombinant derivatives of Pseudomonas aeruginosa exotoxin A containing distinct targeting moieties against gp120. In the present report, we compare the in vitro potency and breadth of these chimeric toxins against multiple clinical HIV-1 isolates, replicating in biologically relevant primary human target cell types. In PBMCs, 3B3(Fv)-PE38 blocked spreading infection by all isolates examined, with greater potency than CD4(178)-PE40. 3B3(Fv)-PE38 also potently inhibited spreading HIV-1 infection in primary macrophages. Control experiments demonstrated that in both target cell types, most of the 3B3(Fv)-PE38 activity was due to selective killing of infected cells, and not merely to neutralization by the antibody moiety of the chimeric toxin. High-dose treatment of rhesus macaques with 3B3(Fv)-PE38 did not induce liver toxicity, whereas equivalent dosage of CD4(178)-PE40 induced mild hepatotoxicity. These findings highlight the potential use of 3B3(Fv)-PE38 for depleting HIV-infected cell reservoirs persisting in the face of HAART.

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Exhibit 6